ON SUBUNIT STRUCTURE OF Ca^{2+}-DEPENDENT ATPase OF SARCOPLASMIC RETICULUM

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1. Introduction

The protein composition of sarcoplasmic reticulum membranes has been extensively characterized by means of polyacrylamide gel electrophoresis in one [1] or two [2] dimensions. It is generally accepted that the protein of mol. wt 100 000 (i.e., Ca^{2+}-ATPase) is the principal protein of sarcoplasmic reticulum. However, monomer and dimer fractions of deoxycholate-solubilized Ca^{2+}-ATPase during isoelectric focusing consistently gave rise to $\geqslant$5 different bands from pI 5.0–5.7 [3]. Later the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum has been shown [4,5] to consist of several different polypeptides of similar molecular weights whose isoelectric points were pI ~5–6. Using the techniques in [4] $\geqslant$3 major protein bands in normal and dystrophic chicken sarcoplasmic reticulum were observed [6]. These three bands were localized between pH 5.0–7.0.

As far as the Ca^{2+}-ATPase of sarcoplasmic reticulum may represent an oligomeric complex of several subunits [7] it was interesting to investigate whether the enzyme is composed of different or identical polypeptides. The results reported here show that the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum is composed of one type of polypeptide that has an apparent isoelectric point of pI 5.91 ± 0.09 and mol. wt ~100 000.

2. Materials and methods

2.1. *Sarcoplasmic reticulum and ATPase preparations*

Rabbit sarcoplasmic reticulum was prepared as in [8]. The delipidated Ca^{2+}-ATPase was isolated and purified as in [9].

Abbreviations: EDTA, ethylene diamine tetraacetic acid disodium salt; DTT, dithiothreitol; SDS, sodium dodecyl sulfate

2.2. *Isoelectric focusing*

The tubes and slabs for isoelectric focusing in first or second dimension consisted of 3.78% acrylamide, 0.22% *N,N'*-methylene bisacrylamide, 8 M or 9.5 M urea, 2% Triton X-100 and 2% ampholytes (Ampholines, LKB, Bromma). The gels were loaded with protein samples prepared in buffer A containing 5% or 10% Triton X-100. Buffer A was composed of 8 M or 9.5 M urea, 1.6 mM EDTA (pH 7.4), 1% DTT and 20 mM Tris (pH 7.4). The cathodal and anodal electrolytes were 20 mM NaOH and 20 mM H_2SO_4, respectively. Electrophoresis was run at 200–400 V for 16–19 h. To determine pH gradients unfixed and unstained rods or strips of slabs were cut in 2 mm slices which were extracted for 2 h by 2.0 ml boiled bidistilled water. For second dimension electrophoresis isoelectric focussed rods or strips were equilibrated for 2 h in buffer B which consisted of 5 mM sodium phosphate (pH 7.0), 1% DTT, 2% SDS, 20% glycerol and 0.002% bromophenol blue and then sealed to the slab with 1% agarose in the same buffer.

2.3. *Gel electrophoresis*

Polyacrylamide gradient gel electrophoresis was performed also both in tubes and in slabs. The gels consisted of 4–10% acrylamide, 0.1–0.25% *N,N'*-methylene bisacrylamide, 4–10% glycerol, 0.1% SDS and 0.1 M sodium phosphate (pH 7.0). The samples of protein were dissolved in buffer B and boiled for 3 min. Upper and lower chamber buffers were, respectively, 0.1 M sodium phosphate (pH 7.0) with or without 0.1% SDS. For second dimension isoelectric focusing strips of slab were soaked for 2 h in a solu-

tion containing 8 or 9.5 M urea, 2% Triton X-100 and 2% ampholytes and then put on a slab.

2.4. *Fixation and staining of gels*

In all cases the gels were fixed first in 50% methanol, then in methanol/acetic acid/water (5:1:4, by vol.) and finally in methanol/sulfosalicylic acid/trichloroacetic acid/water (30:3.45:11.5:55, by vol.). Then the gels were stained with Coomassie brilliant blue R-250 in methanol/acetic acid/water (5:1:4 by vol.) and diffusion destained in a water solution of 15% methanol (v/v) and 7.5% acetic acid (v/v).

3. Results

The Ca^{2+}-ATPase protein accounts for 70–80% of the total protein of rabbit sarcoplasmic reticulum [8]. Isoelectric focussing of the membrane proteins after solubilizing of sample in buffer A containing 5% Triton X-100 gave several main bands (I–VII) with pI 5.60–6.15. The pattern of protein separation (fig.1A) in this case resembles very much that of [4]. As revealed by second dimension electrophoresis in the SDS-system (fig.2A), the polypeptides I,II, III–IV,V,VI and VII with pI 6.15, 6.00, 5.95, 5.85, 5.80 and 5.60 have a mol. wt ~100 000. Fig.2A shows also that in the case of especially overloaded

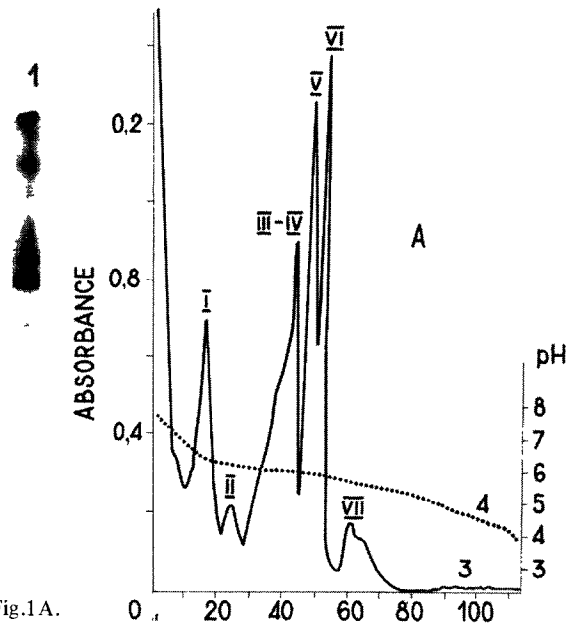

Fig.1A.

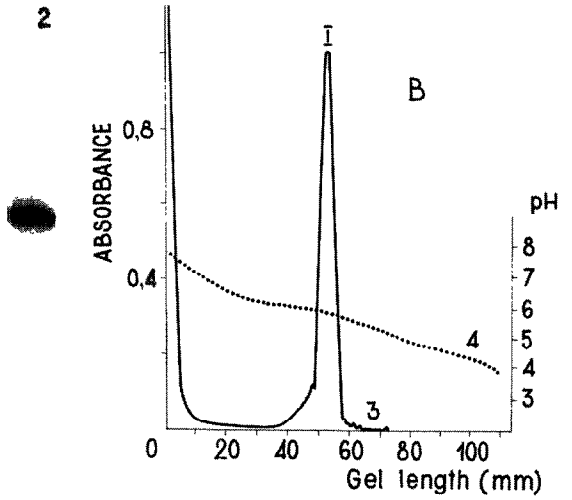

Fig.1. Isoelectric focusing of sarcoplasmic reticulum proteins. (A) Sample 1 was solubilized in buffer A containing 5% Triton X-100, pH gradient was formed of 1.2% pH 3.5–10, 0.4% pH 3–5 and 0.4% pH 5–7. (B) Sample 2 was solubilized in buffer A containing 10% Triton X-100, pH gradient was formed of 0.4% pH 3.5–10, 0.8% pH 4–6 and 0.8% pH 5–7. Gels 1 and 2 were scanned at 560 mm using a Gilford 2400 equipped with the model 2410-S linear transport. Gel 3, A_{560}; gel 4, pH gradient.

gels some faint tracings appear under and above each spot. The tracings are very much alike to a pattern of polypeptides in control sample of sarcoplasmic reticulum.

It appeared reasonable to assume that the concentration of Triton X-100 used by [4] and by us for solubilizing of the protein sample was unsufficient for complete solubilization of the membrane material. Therefore, in the next experiments we increased the effective concentration of Triton X-100 in the sample to 10%. The results of isoelectric focusing and subsequent gel electrophoresis are represented, respectively, in fig.1B and 2B. One can see that the contrary is the case, and the pattern obtained is quite different from that of fig.1A and 2A. During 17 separations under the conditions described we had constantly observed only one major spot corresponding to the Ca^{2+}-ATPase polypeptide; the apparent isoelectric point being estimated at 5.91 ± 0.09 (mean ±SD calculated from 17 expt.).

The same unique spot of Ca^{2+}-ATPase was obtained also in the reverse experiments with sarcoplasmic reticulum (fig.2C) when SDS gel electrophoresis was performed in first dimension and isoelectric focusing

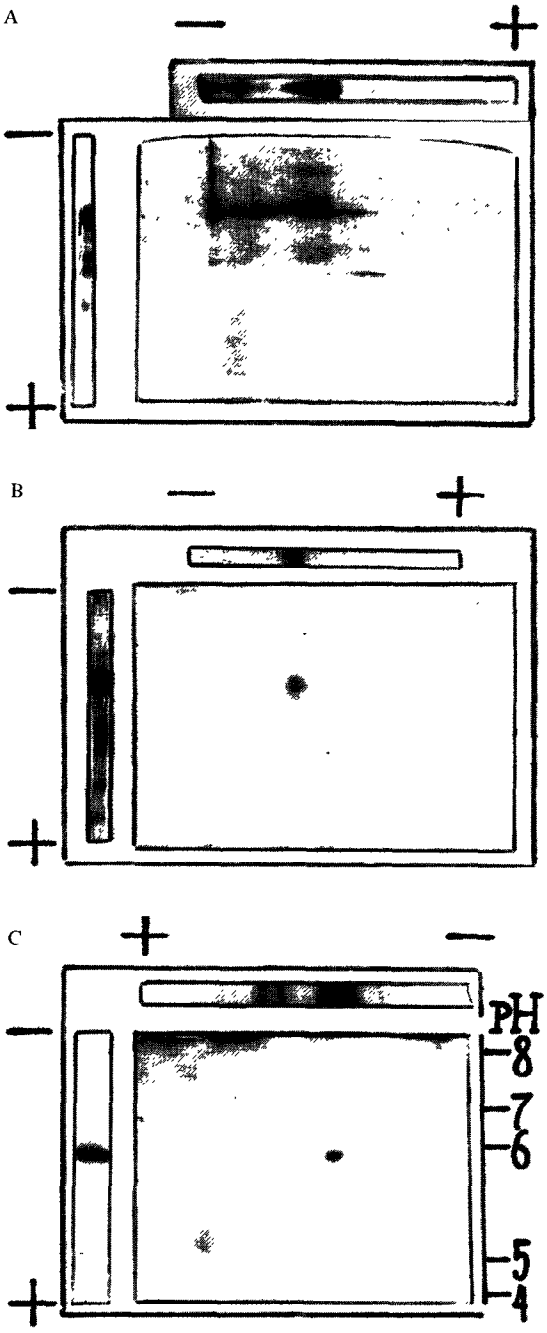

Fig.2.(A) Second dimension electrophoresis in SDS–polyacrylamide gel slabs following isoelectric focusing of gel 1 of fig.1(A). (B) The same for gel 2 of fig.1B. (C) Second dimension isoelectric focusing following SDS–polyacrylamide gel electrophoresis. pH gradient was formed of 2% pH 3.5–10. In all cases upper gels represent first dimension separation. Control samples for second dimension separation are present at the left side of slabs.

was run in the second one. Similar results were obtained also in the direct and the reverse experiments with purified delipidated Ca^{2+}-ATPase. Independently of sequence of the methods applied only a single spot of Ca^{2+}-ATPase could be obtained consistently (fig.3A,B).

4. Discussion

Isoelectric focusing in polyacrylamide gels is a very sensitive method. It allows one to detect very small

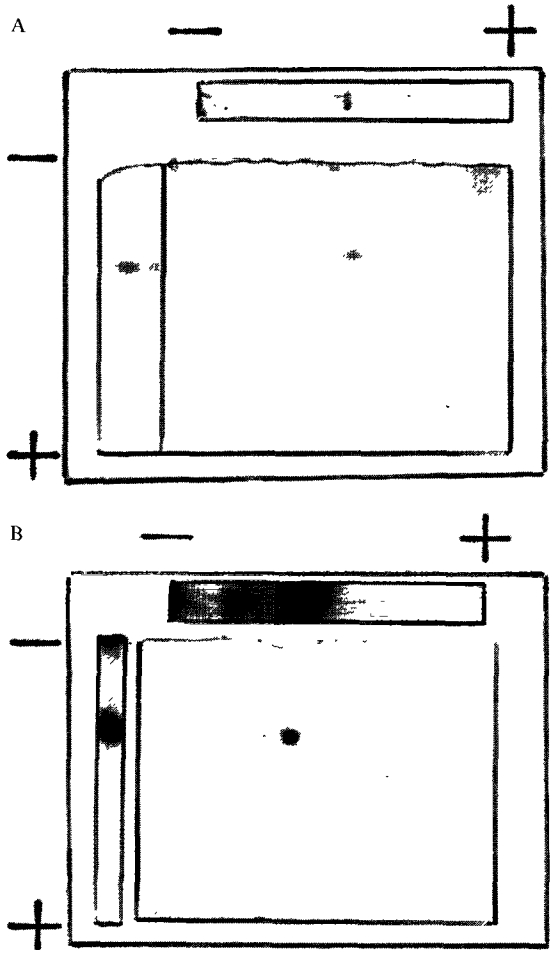

Fig.3. Two-dimensional electrophoresis of delipidated Ca^{2+}-ATPase of sarcoplasmic reticulum. (A) Isoelectric focusing followed by SDS–polyacrylamide gel electrophoresis. (B) SDS–polyacrylamide gel electrophoresis followed by isoelectric focusing. In both cases pH gradient for isoelectric focusing was essentially the same as in fig.1B.

charge differences in proteins. But in some cases the charge differences may be due to exposure of groups buried in one conformation that became available in another conformation or to redistribution of charged groups during conformational transitions. Therefore, it is difficult in many cases to deduce the molecular cause(s) for a multiple band pattern in isoelectric focusing of protein which is apparently homogenous by SDS disc-electrophoresis. But the separation of Ca^{2+}-ATPase protein into several polypeptide zones as in fig.1A and 2A is most likely due to incomplete solubilization of the membrane material in 5% Triton X-100. In such a case, the appearance of bands having different isoelectric migrations may reflect complexes of ATPase and other membrane proteins with various extents of aggregation. This fact re-emphasizes that all methods adopted for the study of soluble proteins (e.g., albumin) must be developed specifically and with due care for the investigation of membrane proteins especially those having large hydrophobic domains.

In so far as the multiple band splitting disappears after solubilization of sarcoplasmic reticulum in 10% Triton X-100 or in 1% SDS the obvious and most attractive conclusion is that the Ca^{2+}-ATPase system of sarcoplasmic reticulum consists of one polypeptide type.

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